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GAS–LIQUID CHROMATOGRAPHY OF THE PURINE AND PYRIMIDINE BASES*

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SUMMARY

The major purpose of this investigation was to develop a quantitative method for the gas–liquid chromatographic analysis of the purine and pyrimidine bases at a macro (100 μg), semimicro (10 μg), and micro (500 ng) level. Included was an evaluation of bis (trimethylsilyl) trifluoroacetamide as the silylating reagent, solvents, and the determination of the optimum silylating and instrumental conditions at each level.

INTRODUCTION

Precise and accurate analytical methods for the analysis of nucleic acid components, including the purine and pyrimidine bases, the nucleosides, and the nucleotides, have long been desired by many researchers in biochemistry, biology, medicine, and other fields of research. Because of the central biological significance of nucleic acids, it is important to know as much as possible about their function, their structure, and their chemical composition.

Nucleic acids are divided into two classes, deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), according to their structure and biological function. The structural differences between RNA and DNA consist of a D-ribose moiety in the RNA molecule and a D-2-deoxyribose moiety in the DNA molecule, the pyrimidine uracil in RNA and the pyrimidine thymine in DNA. One of the biological functions of nucleic acids is involved in the synthesis of enzymatic proteins. The DNA contains a code which directs the synthesis of proteins and acts indirectly through different types of RNA. The messenger RNA, mRNA, takes the genetic code from the DNA in the cell nucleus and transports it to the ribosomes, the site of protein synthesis in the cell. Structural or ribosomal RNA, rRNA, in the ribosomes receives the code from the mRNA and builds the protein with amino acids brought to the ribosomes by transfer RNA, tRNA. Since there are twenty naturally occurring protein amino acids, there is a minimum of twenty tRNA's necessary for protein synthesis.

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The chemical composition of nucleic acids consists of a purine or pyrimidine base attached to the first carbon of the sugar moiety (nucleosides), and a phosphate molecule attached to the sugar at C-5 (nucleotides). The polymer chain is made by attaching the phosphate molecule of one nucleotide to the C-3 of the sugar of another nucleotide. The length of the polymer chain varies, depending on the function and the source of the nucleic acid; some DNA molecules have more than 20,000 nucleotide residues. Because of the importance of nucleic acids in the genetic code, it is necessary to know as much as possible about their composition. To find the base ratios in a nucleic acid, it is first necessary to hydrolyze the polymer into various monomers (purines and pyrimidines, nucleosides, or nucleotides, depending on the type of depolymerization used).

Of interest in many areas of research is the determination of the purine and pyrimidine base ratios, and a number of methods have already been developed. These techniques include ion-exchange chromatography (IEC) by COHN[1], paper chromatography (PC) by VISCHER AND CHARGAFF[2], paper electrophoresis (PE) by GORDON AND REICHARD[3], thin-layer chromatography (TLC) by RANDERATH[4] and gas–liquid chromatography (GLC) by HASHIZUME AND SASAKI[5] and GEHRKE AND RUYLE[6]. All of these methods were developed at a macro (mg) level, and only the GLC methods gave good quantitation.

Since most biological samples are not available in macro quantities, a micro (μg) method, which would give quantitative results, was needed. Since GLC gave good quantitative data at the macro level, and offered speed, selectivity, and sensitivity to an analysis, it was the choice of analysis for the development of a micro method.

However, the purine and pyrimidine bases are not volatile; thus, they must be converted to a volatile derivative before GLC analysis is possible. Various derivatives of the purine and pyrimidine bases have been studied at the macro level; these include the methyl derivative by MACGEE[7], acetyl and isopropylidene derivatives by MILES AND FALES[8], and the trimethylsilyl (TMS) derivative by HASHIZUME AND SASAKI[9] and GEHRKE AND RUYLE[6]. The TMS derivative gave the best results and was the one most likely to work for a micro analysis.

With the development of a new silylating reagent, bis (trimethylsilyl) trifluoroacetamide (BSTFA), by STALLING *et al.*[10], silylation at the micro level was possible. The aims of this investigation were: to evaluate BSTFA as a silylating reagent for the purine and pyrimidine bases; to establish the optimum reaction conditions for quantitative silylation with BSTFA at a macro, semimicro, and micro level; and to investigate the chromatographic properties and instrumental requirements of the derivatized purine and pyrimidine bases.

LITERATURE REVIEW

The analysis of nucleic acid constituents, using chromatographic techniques, has achieved separations due to differences in pK_a values, solubilities, and adsorption coefficients of the various components. Analysis of the purine and pyrimidine bases, nucleosides, and nucleotides by PC has been reviewed by PADR[11], who discussed various solvent systems and detection techniques. COHN[1] reviewed the separation and analysis of the bases, nucleosides, and nucleotides by IEC, and a monograph on TLC

by RANDERATH[4] discusses the uses of TLC for the analysis of these constituents. CIANDI AND ANDERSON[12] have separated 26 purine and 22 pyrimidine derivatives by TLC with a detection limit of 2.0 μg.

In 1962 MILES AND FALES[8] published on the analysis of nucleosides by GLC using acetyl, methyl, and/or isopropylidene derivatives. MACGEE[7], in 1964, investigated the N-methyl derivatives of the purine and pyrimidine bases. Disadvantages included multiple derivatives with as many as four peaks for adenine, but MACGEE[13] was able to obtain some quantitative data and to apply his method to nucleic acids which were hydrolyzed with perchloric acid and cleaned by IE before chromatography.

GLC of the TMS derivatives of nucleosides was first reported by HANCOCK AND COLEMAN[14]. The derivative was prepared by silylation with hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine followed by chromatography on an SE-30 column. Multiple derivatives and unsymmetrical peaks were obtained. HANCOCK[15] later reported on the successful GC of various adenosine derivatives and showed that the TMS derivative could be used for quantitative analysis.

In 1966, HASHIZUME AND SASAKI[5] published on the GLC separation of some of the ribonucleotides using the TMS derivatives. The TMS-nucleotides were prepared with HMDS and TMCS in pyridine, then chromatographed on a DC-430 column. Later, in 1967 and 1968, HASHIZUME AND SASAKI[9,16] reported on the analysis of the TMS derivative of the purine and pyrimidine bases and nucleosides. Using phenanthrene as the internal standard, they were able to obtain quantitative data on the five main bases at the macro level (mg), and they demonstrated the applicability of their method to the analysis of base ratios in an RNA and a DNA sample.

In 1968, GEHRKE AND RUYLE[6] reported a quantitative method for the analysis of the purine and pyrimidine bases using bis (trimethylsilyl) acetamide (BSA) as the silylation reagent. They demonstrated the superiority of BSA to other silylating reagents available at that time and presented data on the derivatization and chromatography of selected nucleosides and nucleotides. Further, they applied their method to the analysis of biological samples of RNA or DNA at the macro level, using a perchloric acid hydrolysis and IE cleanup before derivatization and chromatography on an SE-30 column.

Also in 1968, STALLING *et al.*[10] reported on the synthesis of a new silylating reagent, bis (trimethylsilyl) trifluoroacetamide (BSTFA). BSTFA was shown to be a more powerful silylating reagent than BSA in that it and its breakdown product, MSTFA, were more volatile than BSA and its byproduct, MSA and that the chromatograms obtained with BSTFA were cleaner (fewer extraneous peaks) than those obtained with other silylation reagents. GEHRKE AND RUYLE[6] conducted experiments on the application of BSTFA to the purine and pyrimidine bases and indicated that this reagent, because of its volatility, reactivity, and purity, might serve as a good silylating reagent for the development of a micro method for the analysis of the genetic bases by GLC.

SILYLATION OF MACRO SAMPLES OF THE PURINE AND PYRIMIDINE BASES WITH BSTFA

Experimental

(1) *Apparatus*. An F and M 402 Biomedical Gas Chromatograph (F and M Scientific, Division of Hewlett-Packard, Avondale, Pa.), equipped with dual hydrogen

flame ionization detectors, was used in this study. Drying cylinders packed with $CaSO_4$ and a molecular sieve type 5A were placed in the hydrogen and nitrogen lines to remove water and hydrocarbons. In the air line, a drying cylinder with $CaSO_4$ and a small amount of indicator $CaSO_4$ were placed to remove water.

Area determinations of the chromatographic peaks were made with a Disc Integrator Model 228-A (Disc Instruments, Inc., Santa Anna, Calif.).

For elevated temperature reactions, a magnetically stirred high-temperature oil bath (100–200°), with variable temperature control (±2°), was constructed in this laboratory and used in the initial studies. For later studies, a sand bath with a variable temperature control system (±2°) was used.

Sample aliquots were dried on a 90° sand bath or a 60° hot plate with a stream of compressed nitrogen directed into the reaction vessel.

A Mettler M-5 microgramatic balance (Mettler Instrument Corporation, Hightstown, N.J.) was used to weigh 1.0-mg samples and the samples for preparation of the stock solutions.

A Hamilton 701N 10-μl syringe (Hamilton Company, Whittier, Calif.) was used to introduce the sample into the gas chromatograph.

(2) *Reagents*. The purine and pyrimidine bases (uracil, thymine, cytosine, adenine, and guanine) were obtained from Mann Research Laboratories, New York, N.Y., and were chromatographically pure. BSTFA was purchased in teflon-lined screw cap vials from Regis Chemical Company, 1101 N. Franklin Street, Chicago, Ill. Acetonitrile and acetone were of "Nanograde" purity and were purchased from Mallinckrodt Chemical Works, St. Louis, Mo. The water was doubly distilled from an all-glass distillation apparatus. The other reagents used were of the highest purity available.

(3) *Instrumental and chromatographic conditions*. The chromatographic column was a 10 w/w% GC-SE-30 (straight-chained polymethylsiloxane) on 100–120 mesh Supelcoport (Chromatographic W type). The column materials were packed in glass columns (borosilicate) 1 m long, with an inside diameter of 4 mm, and held in the chromatographic oven by front and back teflon ferrules with normal Swagelok fittings.

The instrumental conditions were a 4-min initial hold, then a 7.5°/min temperature program from 90 to 260°. The detector was operated in the 280–320° range, and the injection heater (flash heater) was at approximately 200°. The gas flow rates were as follows: nitrogen, 40 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min. The attenuation was 1.2×10^{-9} A full scale deflection (a.f.s.).

(4) *Column preparation and conditioning*. The following procedure was used for the preparation of all column packings: (a) Weigh the GC-SE-30 and dissolve in excess chloroform. (b) Weigh the Supelcoport and place in a ridged, ground glass flask. Cover the support with chloroform. (c) Add the dissolved GC-SE-30 to the Supelcoport. (d) Place the mixture on a rotary evaporator, and using a slight amount of vacuum to hold the flask, mix for 15 min. (e) After mixing, slowly evaporate the chloroform by increasing the vacuum and heating the flask at 60°. Note: The chloroform must not be evaporated too quickly or it will "bump", causing some loss of the GC-SE-30. (f) After the chloroform has evaporated, mix the column packing for 30 min under high vacuum and heat at 60° to remove the remaining traces of chloroform.

After the column packing had been prepared, it was packed into a clean, 1-m

glass column with a 4-mm I.D. To ensure uniform packing, the glass column was gently tapped during the addition of the packing. After the column had been prepared, a 1/4-in. plug of "silanized" glass wool was placed in each end.

To give a reproducible column, the following conditioning was used: (a) Place the column in the GC oven. (b) Without carrier gas, "no flow" condition the column for 2 h at 300°. (c) Cool the chromatographic oven to 50° and regulate the carrier gas to 40 ml/min. (d) Program the temperature from 50 to 270° at 2°/min. (e) Condition the column at these settings (270° with a carrier gas flow of 40 ml/min) for 24–48 h.

By following these details for column preparation and conditioning, the columns were reproducible and stable for three months. Signs of column deterioration were an increase in bleed at higher temperatures and a decrease in the response for the TMS-guanine peak. Because the teflon ferrules used to hold the columns in the chromatographic oven tended to co-flow, it was necessary to periodically tighten the Swagelok fittings to prevent the carrier gas from leaking and the column from falling.

Determination of the optimum silylating conditions

GEHRKE AND RUYLE[6] investigated the silylation of the purine and pyrimidine bases using BSA and showed that BSA and BSTFA gave similar results, however, BSTFA gave a cleaner chromatogram and a smaller solvent peak due to the higher volatility of BSTFA and its byproduct MSTFA. GEHRKE AND RUYLE[6] also reported that a 100 molar excess of BSA was required for complete derivatization, and since BSA and BSTFA were similar compounds and gave similar silylation results, a 100 molar excess or greater of BSTFA was used in this study.

(1) *Time and temperature study for maximum derivatization*

The determination of the reaction conditions necessary to achieve complete silylation involved a study of time and temperature. Because each of the major bases, uracil, thymine, cytosine, adenine, and guanine (U, T, C, A, G) might have different optimum silylation times and temperatures, it was necessary to determine the reaction conditions for each base independently and to choose the conditions that gave the best results for all five bases.

Sample preparation. Stock solutions of uracil and cytosine, thymine and guanine, and adenine and guanine were prepared by dissolving 20 mg of the base in 100 ml of 7.5 N NH_4OH. Guanine was analyzed in duplicate because initial experiments had shown it to be the most difficult base to silylate. A 5.0-ml aliquot (*ca.* 1.0 mg of each base) was quantitatively transferred into a 16 × 75 mm culture tube with a teflon-lined screw cap. The sample was dried on a 90° sand bath with a stream of pure compressed nitrogen. After the sample was dry, 1.0 mg of phenanthrene (the internal standard) in 5.0 ml of acetone was added. The acetone was removed with an IR lamp and a stream of compressed nitrogen.

Derivatization. The dried sample containing *ca.* 1.0 mg of each of two bases (U and C, T and G, and A and G) was silylated with 1.0 ml BSTFA and 1.0 ml acetonitrile (CH_3CN). Duplicate samples were heated for 15, 45, 60, and 120 min at temperatures of 125° and 150°. The silylated samples were immediately cooled to room temperature, and 2–4 µl were injected into the gas chromatograph.

Calculation. The relative molar response ($RMR_{B./I.S.}$) of the TMS base to phenanthrene was calculated as follows:

$$RMR_{B./I.S.} = \frac{\dfrac{Area_{B.}}{Grams_{B.}/MW_{B.}}}{\dfrac{Area_{Phen.}}{Grams_{Phen.}/MW_{Phen.}}} = \frac{\dfrac{A_{B.}}{Moles_{B.}}}{\dfrac{A_{I.S.}}{Moles_{I.S.}}}$$

where

$Area_{B.}$ = area chromatographic peak for the base

$Area_{Phen.}$ = area chromatographic peak for phenanthrene (I.S.)

MW = molecular weight

The relative molar response, $RMR_{B./I.S.}$, was calculated for each base and plotted as a function of reaction time.

(2) *Reproducibility and stability of silylation using BSTFA*

The optimum silylating conditions for the five bases (U, T, C, A, and G) using BSTFA were chosen from the previous experiments (150° for 15 min). These reaction conditions were then used to check the reproducibility of silylation and the stability of the TMS bases with time.

Reproducibility of silylation. A stock solution containing 20.0 mg each of U, T, C, A, and G was prepared, and six 5.0-ml aliquots were pipetted and dried. The samples were silylated with 2.0 ml BSTFA and 2.0 ml acetonitrile at 150° for 15 min. The results are given in Table I, including the standard deviation for the $RMR_{B./I.S.}$ values for the five bases.

TABLE I

RELATIVE MOLAR RESPONSE OF THE TMS DERIVATIVE OF THE PURINE AND PYRIMIDINE BASES[a]

Compound	*Retention temperature (°C)*	$RMR_{B./Phen.}$[b]	*S.D.*[c]
Uracil	120	0.63	0.004
Thymine	130	0.67	0.007
Cytosine	144; 162	0.67[d]	0.004
Adenine	188	0.66	0.011
Guanine	212	0.75	0.004
Phenanthrene (I.S.)	172	1.00	

[a] Flame ionization detector used. Silylated with BSTFA at 150° for 15 min.
[b] RMR determined from six independent determinations.
RMR = relative molar response to phenanthrene as I.S.

$$RMR_{B./I.S.} = \frac{A_{B.}/Moles_{B.}}{A_{I.S.}/Moles_{I.S.}}$$

[c] Standard deviation calculated from at least four independent determinations. Average R.S.D. = 0.9%.
[d] Two peaks were obtained for cytosine, each was integrated independently, then added to calculate RMR.

Stability of the TMS base with time. Two of the samples in the previous experiment were used to determine stability of the silylated bases. Each sample was run on five successive days at the same chromatographic conditions. The $RMR_{B./I.S.}$ values for each sample were calculated, and the data are presented in Table II.

TABLE II

RELATIVE MOLAR RESPONSE AND STABILITY OF THE TMS DERIVATIVE OF THE PURINE AND PYRIMIDINE BASES AS A FUNCTION OF TIME

Sample[b,c]	*Base*	$RMR_{B./I.S.}$[a]				
		Initial	*2 days*	*4 days*	*5 days*	*Av.*
1	Uracil	0.63	0.61	0.63	0.65	0.63
	Thymine	0.67	0.63	0.64	0.65	0.65
	Cytosine[d]	0.67	0.63	0.63	0.64	0.64
	Adenine	0.64	0.62	0.63	0.61	0.63
	Guanine	0.74	0.72	0.72	0.71	0.72
2	Uracil	0.63	0.66	0.67	0.68	0.66
	Thymine	0.68	0.69	0.68	0.69	0.68
	Cytosine[d]	0.69	0.66	0.69	0.66	0.67
	Adenine	0.66	0.66	0.66	0.65	0.66
	Guanine	0.75	0.77	0.77	0.77	0.76

[a] $RMR_{B./I.S.} = \frac{A_{B.}/Moles_{B.}}{A_{I.S.}/Moles_{I.S.}}$.

[b] Samples were used in precision study.

[c] Flame ionization detector used. Silylated with BSTFA at 150° for 15 min.

[d] Two peaks were obtained for cytosine, each was integrated independently, then added to calculate RMR.

Determination of impurity at 124° and loss of internal standard phenanthrene

While determining the optimum conditions for silylation of the purine and pyrimidine bases using BSTFA, a large impurity eluted at 124° (just before the uracil peak). Also, some samples were lost due to a reduction in the phenanthrene peak. Thus, it was necessary to remove the 124° impurity from the samples as it interfered with the uracil peak, and to determine the reason for loss of internal standard.

The impurity peak at 124°. The analysis of the impurity eluted from the chromatogram at 124° consisted of finding the source of contamination and eliminating it from the samples. The only possible sources of contamination were the stock solution solvent (7.5 *N* NH_4OH), the BSTFA, the acetonitrile, or an impurity in the bases. A reagent blank (1.0 ml BSTFA and 1.0 ml acetonitrile heated at 150° for 15 min) did not contain the impurity, and the bases, when weighed directly into the reaction tube before silylation, were also pure. When 5.0 ml of 7.5 *N* NH_4OH were dried and silylated, the impurity at 124° was present. By spiking the sample, the impurity at 124° was found to be TMS-ammonium phosphate.

To remove this impurity, a new stock solution solvent was needed that was pure, volatile, and would dissolve the bases. 3 *N* HCl met all of these conditions, and on analysis gave a clean chromatogram with reproducible $RMR_{B./I.S.}$ values.

Determination of phenanthrene (I.S.) loss. The determination of loss of phenanthrene consisted of finding the cause of the loss and removing this step in the sample preparation. Since adenine was eluted just after phenanthrene, it was used to check the $RMR_{B./I.S.}$ values in this experiment. A stock solution of 3.3 mg of adenine in 100 ml of 3 *N* HCl (100 μg/3.0 ml) was prepared, and twelve 3.0-ml samples (*ca.* 0.10 mg adenine) were pipetted and taken to dryness. Also, three stock solutions of phenan-

threne in acetone were prepared: 2.5 mg/25 ml acetone (0.100 mg/1.0 ml), 1.25 mg/25 ml acetone (0.100 mg/2.0 ml), and 3.33 mg/100 ml acetone (0.100 mg/3.0 ml). Each stock solution of phenanthrene was used with four of the adenine samples. The acetone was evaporated from the samples with an IR lamp and a stream of pure nitrogen gas. Two of the samples with each level of internal standard stock solution were placed directly under the IR lamp, and two were heated indirectly. After drying, the samples were silylated with 0.2 ml BSTFA and 0.2 ml acetonitrile at 150° for 15 min. The $RMR_{A/Phen.}$ value for each sample was calculated, and the results are presented in Table III.

TABLE III

LOSS OF PHENANTHRENE (I.S.) ON EVAPORATION

Phenanthrene in acetone	*$RMR_{A/Phen.}$* [a]			
	Indirect heating		*Direct heating*	
	1	*2*	*3*	*4*
0.100 mg/1.0 ml	0.67	0.68	0.79	0.81
0.100 mg/2.0 ml	0.85	0.80	1.02	1.15
0.100 mg/3.0 ml	1.27	1.78	1.54	2.25

Drying method	*$RMR_{A/Phen.}$* [a]	
	1	*2*
Nitrogen only, at room temperature	0.716	0.745
IR lamp and nitrogen	0.603	0.618
IR lamp and nitrogen for 30 min	very small Phen. peak	very small Phen. peak
90° sand bath and nitrogen	very small Phen. peak[b]	very small Phen. peak[c]

[a] $RMR_{A/Phen} = \frac{A_A/Moles_A}{A_{Phen.}/Moles_{Phen.}}$.

[b] Sample removed from sand bath immediately after drying.

[c] Sample left in sand bath 10 min after drying.

As a further check on the phenanthrene loss, eight more adenine samples were prepared. One milliliter of acetone containing 0.100 mg of phenanthrene (I.S.) was added to each sample. The acetone in two of the samples was evaporated by a stream of pure nitrogen at room temperature, two samples by an IR lamp and a stream of nitrogen gas, and two samples by an IR lamp and a stream of nitrogen gas, then left under the lamp for an additional 30 min. The acetone in the last two samples was evaporated on a 90° sand bath with a stream of pure nitrogen, and one of the samples was left in the sand bath for an additional 10 min. The samples were silylated and chromatographed as before, and the $RMR_{A./Phen.}$ calculated. The data are given in Table III.

SILYLATION OF SEMIMICRO AND MICRO SAMPLES OF BASES WITH BSTFA

Experimental

Apparatus. See also *Experimental* on p. 47. A semimicro anb micro culture tube, shown in Fig. 1, were used for the silylation of samples.

A Hamilton 710N 100-μl syringe (Hamilton Company, Inc., Whittier, Calif.) was used to pipet the samples.

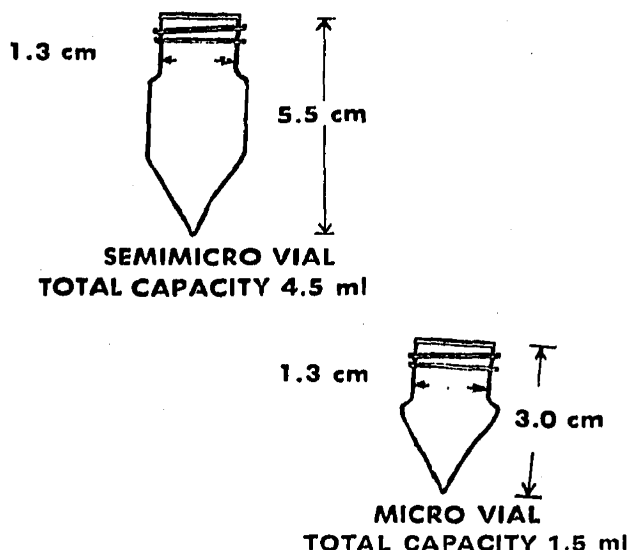

Fig. 1. Reaction vials for derivatization of semimicro and micro samples.

Reagents. Sodium sulfate (A.R. grade), calcium sulfate (A.R. grade), and calcium chloride (A.R. grade) were obtained from Fisher Scientific Company St. Louis, Mo., dichloroethane (ACS grade) and methylene chloride "Nanograde" from Mallinckrodt Chemical Works St. Louis, Mo.

Triply distilled water from an all-glass apparatus was used. The other reagents used were of the highest purity available.

Instrumental and chromatographic conditions. The attenuation for the semimicro samples was 3.2×10^{-10} a.f.s. and for the micro samples 8.0×10^{-11} a.f.s. The chromatographic columns used were conditioned for two weeks at 270° with a carrier flow of 40 ml/min. This long conditioning time was needed because of the bleed encountered when micro analyses were being performed.

Silylation of semimicro samples of the purine and pyrimidine bases with BSTFA

Once the analysis of macro (*ca.* 1.0 mg and 0.1 mg each of U, T, C, A, and G) had been evaluated, it was necessary to determine the best silylation conditions of the bases with BSTFA at a semimicro level (*ca.* 10 μg each), so that an analysis of RNA or DNA can be made when limited sample is available.

(1) *Stock solution solvent for the bases.*

The stock solution solvent (3 *N* HCl) used for the macro samples of the bases was found to be unsatisfactory in that the $RMR_{B./I.S.}$ value for guanine changed when the stock solution was allowed to stand for a few days. The $RMR_{G/Phen.}$ value

for a fresh stock solution would gradually decrease with time until the guanine peak completely disappeared from the chromatogram. Thus, it was necessary to evalute other solvents for preparation of the stock solution. The solvent needed to have the following qualities: purity, volatility, stability, and should be one in which the purine and pyrimidine bases were soluble at a low level without affecting their structure or chemical properties. Many solvents had the first three qualities; and in order to check their ability to dissolve the bases, 1.0 mg of each base was weighed and placed in a 100-ml volumetric flask. The solvents studied were: acetonitrile, acetone, dichloromethane, dimethylformamide, dimethyl sulfoxide, methanol, dichloroethane, chloroform, ethyl acetate, benzene, and organic and inorganic acids and bases. The solvent was added, heated, and mixed thoroughly in an ultrasonic mixer.

(2) *Experiments on silylating and instrumental conditions for semimicro samples.* To obtain the proper chromatographic peak sizes for analysis of semimicro samples (*ca.* 10 μg of each base) it was necessary to change either the amount of silylating reagents or the instrumental settings, or both, as presented for the analysis of macro samples. Thus, the amount of silylating reagents, the instrumental settings, and both, were evaluated independently.

Concentration of silylating reagents. Four samples containing *ca.* 10 μg of each base were quantitatively pipetted from the stock solution (3.33 mg of each base/100 ml 0.1 N HCl; 10 μg of each base/300 μl) and dried as described for macro samples under *Sample preparation* on p. 49. Each sample was silylated with 200 μl BSTFA and 200 μl acetonitrile, containing *ca.* 10 μg phenanthrene, at 150° for 15 min. After silylation the samples were concentrated to about 50 μl by flushing with a stream of pure nitrogen gas at room temperature. 3–4 μl of each sample were chromatographed at the instrumental settings developed for macro samples.

Instrumental settings. Four samples were prepared and silylated as discussed above. Each sample was chromatographed at attenuations of 4×10^{-11}, 8×10^{-11}, 1.6×10^{-10}, and 3.2×10^{-10} a.f.s.

Amount of silylating reagents and instrumental settings. Four samples were prepared as above and silylated with the following amounts of BSTFA and acetonitrile at 150° for 15 min: Sample 1, 150 μl and 150 μl; Sample 2, 100 μl and 100 μl; Sample 3, 75 μl and 75 μl, and Sample 4, 50 μl and 50 μl. Each sample was chromatographed at an attenuation that would give approximately 3/4 f.s.d. on the strip chart recorder for the largest peak.

(3) *Effect of low salt concentration on the silylation of semimicro samples*

The reagents used in the preparation of samples were dried with various drying agents, thus, it was necessary to determine the effect of these drying agents on silylation and chromatography of the bases. Three salts, $CaCl_2$, Na_2SO_4 and $CaSO_4$ (all used as drying agents), were checked by adding *ca.* 10 μg of the salt in 100 μl of water to a previously dried sample of 10 μg of each base. The sample was again dried on a 60° hot plate with a stream of pure nitrogen gas. The last traces of water were removed by azeotroping with methylene chloride. The samples were silylated with 100 μl BSTFA and 100 μl acetonitrile at 150° for 15 min. Duplicate samples for each salt were run.

(4) *Effect of low concentrations of water on the silylation of semimicro samples*

Since many of the reagents used for the analysis of the purine and pyrimidine

bases were hygroscopic, the effect of small quantities of water on the silylation of the bases was investigated. Four solutions of acetonitrile were prepared containing 0.5 µl, 1.0 µl, 2.0 µl, and 5.0 µl water per 100 µl acetonitrile. Each water–acetonitrile solution was used for the silylation of two previously dried base samples containing 10.0 µg of each. The results are presented in Table IV.

TABLE IV

THE EFFECT OF LOW CONCENTRATIONS OF WATER ON THE SILYLATION OF THE PURINE AND PYRIMIDINE BASES

Water added (µl/100 µl acetonitrile)	*RMR$_{B./I.S.}$*[a]				
	U	*T*	*C*[b]	*A*	*G*
0.0	0.53	0.58	0.53	0.61	0.66
0.5	0.53	0.60	0.53	0.61	0.61
1.0	0.52	0.57	0.49	0.58	0.61
2.0	0.58	0.63	0.49	0.65	0.70
5.0	0.56	0.57	0.41	0.54	0.17

[a] $RMR_{B./I.S.} = \dfrac{A_{B.}/Moles_{B.}}{A_{I.S.}/Moles_{I.S.}}$.

RMR$_{B./I.S.}$ values represent the average of two independent runs.

[b] Two peaks for cytosine were integrated separately and summed for total area.

Silylation of micro samples of the purine and pyrimidine bases with BSTFA

Because the analysis of many biological samples of nucleic acids would be at a micro level, a method for the silylation and chromatography of the purine and pyrimidine bases at a micro level (500 ng of each base) was needed. As it had been necessary to alter both the amount of silylating reagents and the instrumental settings for the analysis of semimicro quantities of the bases, the analysis of samples in the µg-range would require similar changes in reagent amounts and instrumental settings.

(1) *Amount of silylating reagents for micro samples*

Because the amount of silylating reagents used for micro samples would be approximately 100 µl, a smaller silylation tube (Fig. 1) was made to prevent the complete vaporization of the silylating reagents when the sample was heated at 150°.

Acetonitrile as the silylation solvent. The analysis of semimicro quantities of the bases had been accomplished by lowering the amount of silylating reagents and changing the instrumental attenuation, thus, this approach was used for the analysis of micro samples. A new stock solution (1.0 mg of each base/100 ml 0.1 *N* HCl) was prepared, and four 50-µl samples (*ca.* 500 ng each) were pipetted and dried. Each sample was silylated with 50 µl BSTFA and 50 µl acetonitrile at 150° for 15 min. 5 µl of each sample were analyzed at an attenuation of 4×10^{-11} a.f.s.

Evaluation of other solvents for silylation. Acetonitrile was unsatisfactory as a silylating solvent for micro samples because it bled slowly from the GLC column, thus it was necessary to find a new silylating solvent for the micro samples. The solvent must be eluted from the column prior to the uracil peak, to give reproducible RMR$_{B./I.S.}$ values for all of the bases and not affect the chromatography of the bases. Methylene chloride, dichloroethane, and benzene were evaluated by silylating pre-

viously prepared micro samples with 50 µl BSTFA and 50 µl of each solvent at 150° for 15 min.

Evaluation of dichloroethane. Since dichloroethane gave promising results, further studies were conducted to determine the optimum silylating conditions of dichloroethane and BSTFA. Fourteen micro samples were pipetted, dried, and silylated with 35 µl BSTFA and 35 µl dichloroethane. Six samples were silylated at 150° for times of 1, 2, 3, 4, 5, and 6 h; four samples were silylated at 125° for times of 2, 4, 5 and 6 h; and four samples were silylated at 175° for times of 1, 2, 3, and 4 h. 5 µl of each sample were chromatographed at an attenuation of 8×10^{-11} a.f.s. The results are presented in Table V.

TABLE V

EFFECT OF TIME AND TEMPERATURE ON THE SILYLATION OF BASES AT THE MICRO LEVEL USING DICHLOROETHANE AS SOLVENT

Time H	*Temperature (°C)*	$RMR_{B./I.S.}$ [a]				
		U	*T*	*C* [b]	*A*	*G*
1	150	0.75	0.80	0.65	0.35	0.09
2	150	0.65	0.75	0.63	0.54	0.36
3	150	0.64	0.73	0.66	0.62	0.40
4	150	0.63	0.78	0.67	0.64	0.47
5	150	0.74	0.80	0.62	0.54	0.23
6	150	0.66	0.71	0.62	0.58	0.29
2	125	0.64	0.71	0.63	0.31	0.19
4	125	0.61	0.69	0.52	0.34	no peak
5	125	0.58	0.69	0.60	0.42	0.15
6	125	0.66	0.75	0.60	0.38	no peak
1 [c]	175	—	—	—	—	—
2	175	0.65	0.77	0.60	0.49	no peak
3	175	0.62	0.72	0.60	0.50	0.21
4	175	0.67	0.73	0.58	0.52	0.23

[a] $RMR_{B./I.S.} = \dfrac{A_{B.}/Moles_{B.}}{A_{I.S.}/Moles_{I.S.}}$.

[b] The two cytosine peaks were integrated separately and summed for total area.

[c] Sample lost; silylation tube cap failure.

Evaluation of an acetonitrile–dichloroethane mixture as the silylating solvent. Because the guanine peak was low when dichloroethane was used as the silylating solvent, a mixture of acetonitrile and dichloroethane was evaluated at the semimicro and micro levels. Four semimicro samples were pipetted, dried, and silylated with 100 µl BSTFA and the following solvents: Sample 1, 100 µl acetonitrile; Sample 2, 75 µl acetonitrile and 25 µl dichloroethane; Sample 3, 50 µl acetonitrile and 50 µl dichloroethane; and Sample 4, 25 µl acetonitrile and 75 µl dichloroethane. Four micro samples were also analyzed at the same solvent ratios (1:0, 3:1, 1:1, and 1:3) with a total volume of 100 µl. All samples were heated at 150° for 15 min and then chromatographed.

(2) *Evaluation of optimum silylating temperature and time for micro samples*

Since the silylating solvent used for micro samples had been changed from acetonitrile to a 1:1 mixture of acetonitrile and dichloroethane, it was necessary to determine the optimum silylating temperature and time for these reagents. Since the optimum silylating conditions for acetonitrile and dichloroethane alone had already been determined (acetonitrile alone at 150° for 15 min and dichloroethane alone at 150° for 3 h) 150° was selected as the silylating temperature. Six micro samples were pipetted and dried, and 50 μl BSTFA, 25 μl acetonitrile, and 25 μl dichloroethane were added. Two of each of the samples were heated at 150° for 15, 45, and 60 min. The samples were then analyzed by GLC.

To check the reproducibility of silylation of micro samples, five samples were prepared and silylated at 150° for 30 min. The average $RMR_{B./I.S.}$ and the standard deviation for each base were calculated.

RESULTS AND DISCUSSION

Macro samples using BSTFA

Optimum silylating conditions. Initial investigations using BSTFA as a silylating reagent, and studies by GEHRKE AND RUYLE[6], showed that uracil, thymine, adenine, and guanine gave single, symmetrical, reproducible peaks with silylating conditions of 150° for 45 min (optimum silylating conditions developed by GEHRKE AND RUYLE for BSA). Cytosine gave two symmetrical peaks, on silylation with BSTFA, and the $RMR_{C/Phen.}$ was calculated by integrating each peak separately and adding the areas to obtain the total area for cytosine. Since the response for each peak of cytosine was dependent on the time and temperature of silylation, summation of the areas gave a constant $RMR_{C/Phen.}$ for any conditions.

Because BSTFA was reported by STALLING *et al.*[10] to be a more powerful silylating reagent than BSA, a study of reaction time and temperature was undertaken to determine the conditions necessary to obtain maximum yield of derivative for each base in minimum time. The $RMR_{B./I.S.}$ for U, T, C, A, and G, as a function of silylation time at temperatures of 125° and 150°, are presented in Fig. 2–6. All the bases except thymine gave higher $RMR_{B./I.S.}$ values at 150° with the highest

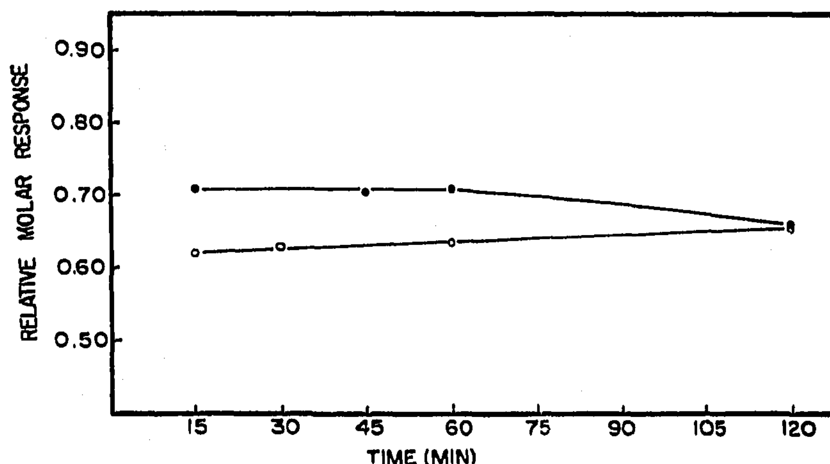

Fig. 2. BSTFA silylation of uracil ●—●, 150° silylation; ○—○, 125° silylation.

values being at a time of 15 min. At 125°, cytosine was not completely silylated before 60 min. From these experiments, the optimum silylating conditions for the purine and pyrimidine bases with BSTFA were determined as 150° for 15 min in a closed reaction vial.

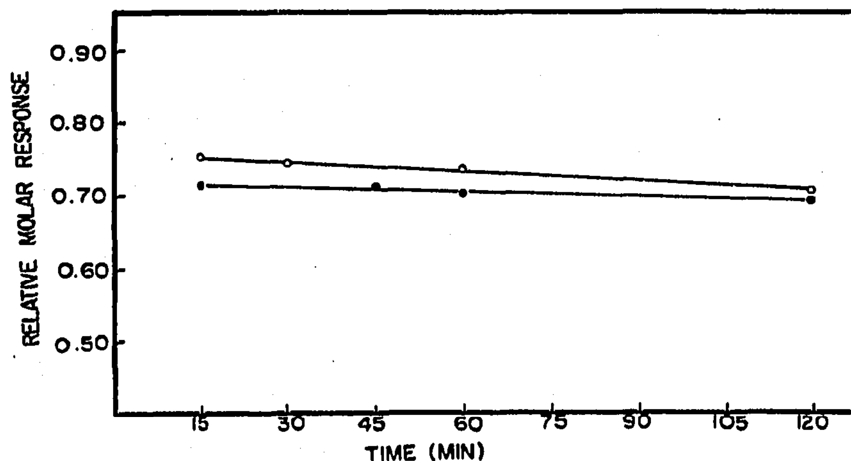

Fig. 3. BSTFA silylation of thymine ●—●, 150° silylation; ○—○, 125° silylation.

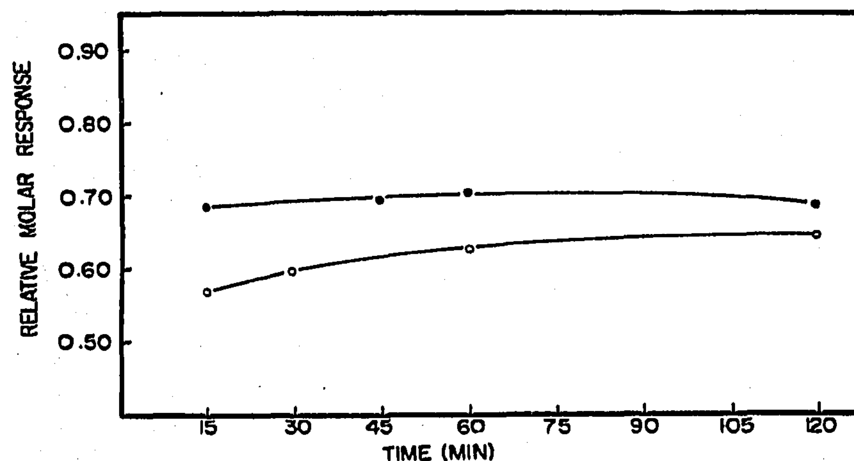

Fig. 4. BSTFA silylation of cytosine ●—●, 150° silylation; ○—○, 125° silylation.

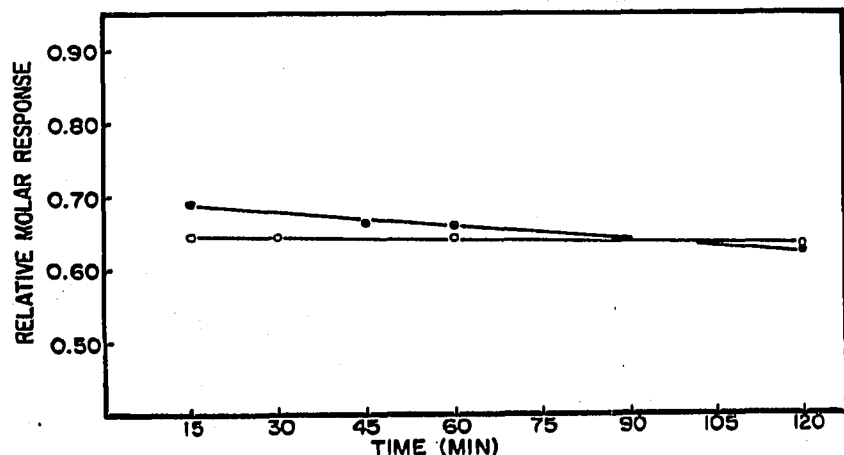

Fig. 5. BSTFA silylation of adenine. ●—●, 150° silylation; ○—○, 125° silylation.

Reproducibility and stability. The relative molar response, $RMR_{B./I.S.}$, and the retention temperature of the purine and pyrimidine bases were determined using the above selected conditions. These data, along with the standard deviation for the $RMR_{B./I.S.}$ of each base, are presented in Table I.

The stability of the silylated bases over a period of five days is given in Table II. The $RMR_{B./I.S.}$ for each base was stable up to five days within the limits of experi-

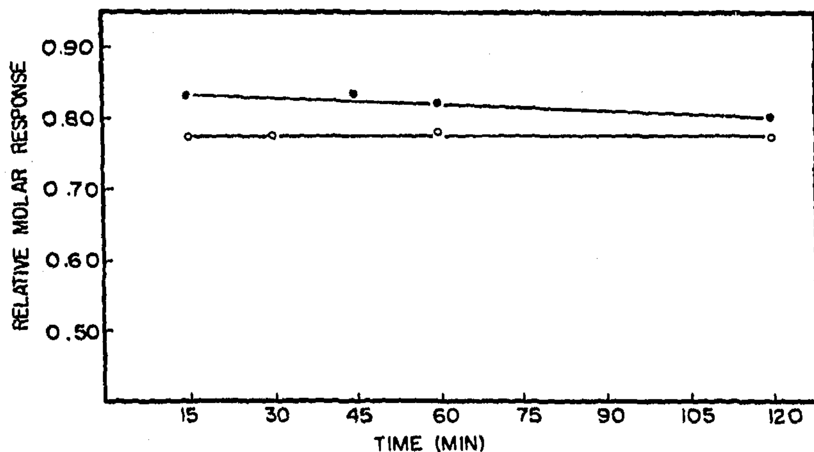

Fig. 6. BSTFA silylation of guanine. ●—●, 150° silylation; ○—○, 125° silylation.

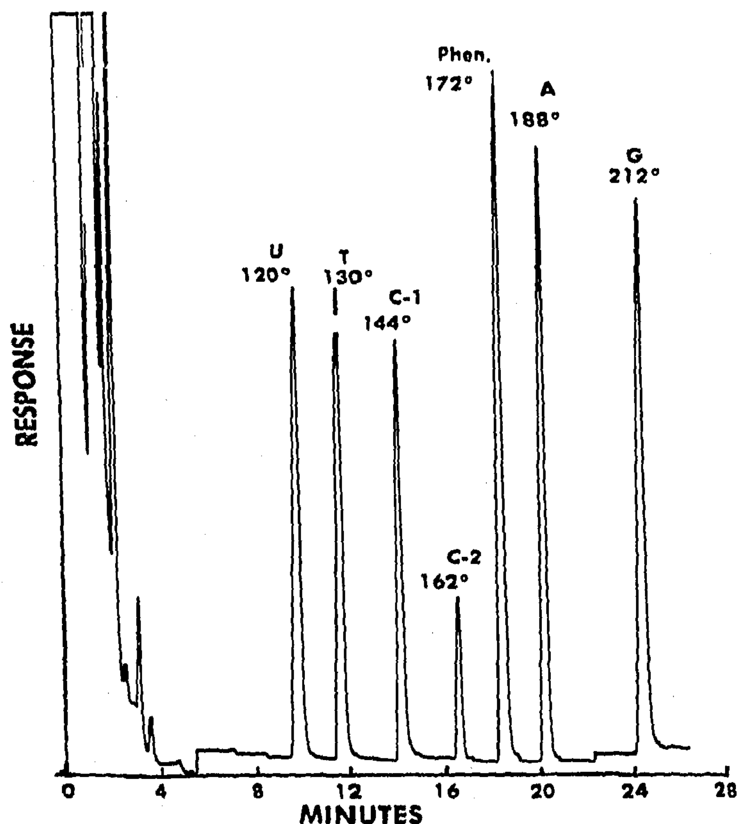

Fig. 7. GLC analysis of the purine and pyrimidine bases. Chromatographic conditions: 7.5°/min temperature program from 90° to 200° with a delay of 4 min; flash heater temperature, 260°; attenuation, 10 × 32. Column: 1 m × 4 mm I.D. Pyrex, 10% SE-30 on Supelcoport (100–120 mesh). Silylation conditions: 100 µg each in reaction vial; 200 µl BSTFA; 200 µl acetonitrile; at 150° for 15 min; 4.0 µl (*ca*. 1 µg of each) were injected.

mental error. A typical chromatogram of the bases is shown in Fig. 7. This chromatogram represents 1 μg of each base injected. The chromatographic conditions are given in the legend to the figure.

Phenanthrene loss. The $RMR_{A/Phen.}$ values shown in Table III for the direct and indirect evaporation of the acetone indicated that the phenanthrene internal standard was subliming when subjected to heat. Further studies to confirm this sublimation (Table III) showed that any heating of phenanthrene caused sublimation and even evaporation at room temperature caused some loss. Thus, to prevent the sublimation of phenanthrene, a stock solution of phenanthrene in acetonitrile, the silylating solvent, was prepared. Since the acetonitrile solvent was added just prior to derivatization, no loss of phenanthrene occurred.

Semimicro samples

Stock solution solvent. An evaluation of solvents for the preparation of the best stock solutions for the purine and pyrimidine bases consisted of testing the solubility of the bases in a number of solvents such as: acetonitrile, acetone, dichloromethane, dimethylformamide, dimethylsulfoxide, methanol, dichloroethane, chloroform, ethyl acetate, and benzene. None of these solvents dissolved all of the bases at the 1.0 mg/100 ml level. The organic acids and bases checked were: 1.0 *N*, 3.0 *N*, and 6.0 *N* formic acid; 1.0 *N*, 3.0 *N*, and 6.0 *N* acetic acid; and pyridine; and none of these would completely dissolve all of the bases. However, it was observed that all of the bases were soluble in 3.0 *N* HCl, but stability over a period of time was a problem. By lowering the concentration of HCl to 0.1 *N*, the bases were still soluble, but not at a lower HCl concentration. The stability of the bases in 0.1 *N* HCl was determined and found to be greater than two months, thus 0.1 *N* HCl was considered satisfactory as a stock solution solvent.

Silylating conditions and instrumental settings. A change in relative amounts of silylating reagents for semimicro samples showed that no apparent loss of the silylated bases occurred, however, the chromatograms for many of the samples contained several large extraneous peaks. These peaks were attributed to impurities in the BSTFA.

When the attenuation for the analysis of semimicro samples was lowered to give chromatographic peaks, one-half to three-fourths full scale, the baseline was unstable, and the bleed rate was enhanced. At higher attenuations, the chromatographic peaks were not large enough for reproducible $RMR_{B./I.S.}$ values.

When both the amounts of silylating reagents and the attenuation were changed, good results were obtained for samples silylated with 100 μl BSTFA and 100 μl acetonitrile and chromatographed at an attenuation of 3.2×10^{-10} a.f.s. Also, the chromatographic peaks were large enough to obtain reproducible $RMR_{B./I.S.}$ values, and the baseline was fairly stable. A typical chromatogram for a semimicro analysis of the bases is shown in Fig. 8. This chromatogram represents 200 ng of each base injected.

The effect of salt. The silylation of the bases in the presence of small amounts of salts did not greatly affect the $RMR_{B./I.S.}$ values for any of the bases. With 10 μg Na_2SO_4 or 10 μg $CaSO_4$ added to a 10-μg sample of each base, no change in the $RMR_{B./I.S.}$ was noticed; however, with 10 μg $CaCl_2$, the guanine peak was slightly reduced. These results indicated that drying the solvents with these salts would not affect the silylation or chromatography of the bases. But, since the salts could have

an adverse effect on the silylation reaction, care was taken to prevent too much salt from entering into the reaction vial.

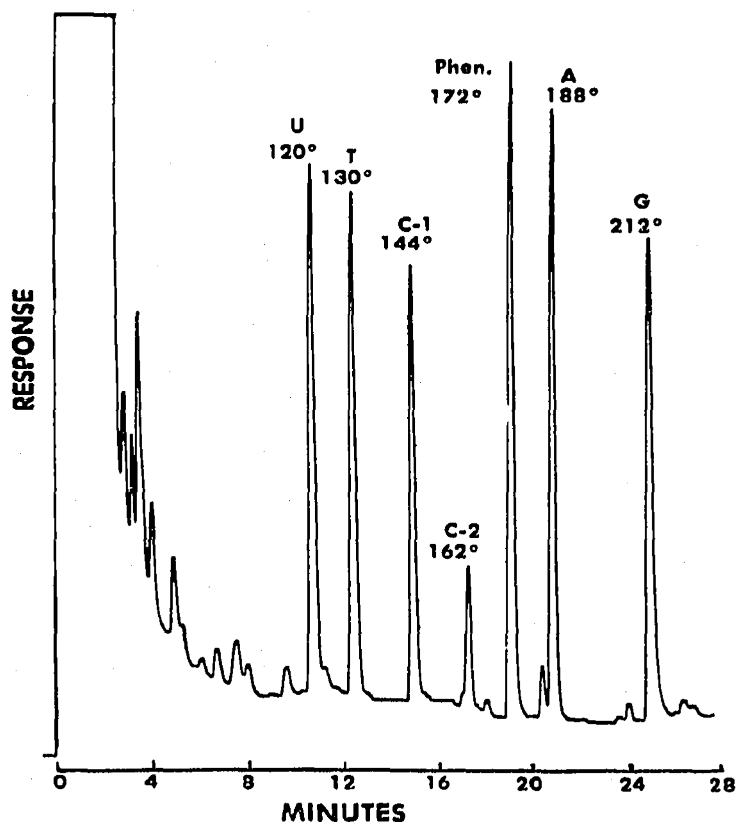

Fig. 8. GLC analysis of the purine and pyrimidine bases. Chromatographic conditions and column, see the legend to Fig. 7. Attenuation, 10 × 8. Silylation conditions: 10 μg each in reaction vial; 100 μl BSTFA; 100 μl acetonitrile; at 150° for 15 min; 4 μl (*ca.* 200 ng of each base) were injected.

Effect of water. The effect of water on the silylation of the bases is shown in Table IV. The silylated bases were stable up to a concentration of 5.0 μl water in 200 μl total volume. Cytosine was the first base to show the effect of water with the lowering, and finally the loss, of its second peak. The $RMR_{C/Phen.}$ did not show a corresponding change because the first peak increased relative to the decrease of the second peak. With 5.0 μl water added, both cytosine and guanine were adversely affected, and a corresponding lowering of their $RMR_{B./I.S.}$ values was noted.

These results show that a low concentration of water could be present during silylation without affecting the chromatography. But, since water at a higher concentration did affect the silylated bases, it was necessary to keep the samples anhydrous before silylation to obtain reproducible data for all the bases.

Micro samples

Silylation solvent. The results of using acetonitrile as the silylating solvent for micro samples showed that the bases could be silylated at this level, but the chromatography was poor. Acetonitrile was retarded in eluting from the column giving a very broad peak with a tail to 140°. This large tailing solvent peak caused the peaks for uracil and thymine to be lost in some samples and on the tail in others.

To find a solvent that had good chromatographic characteristics, methylene

chloride, dichloroethane, and benzene were evaluated. These solvents gave good baselines and good solvent peaks, but the peak for guanine was lost when methylene chloride and benzene were used as the silylating solvent, and reduced response for guanine was obtained with dichloroethane .

Because dichloroethane showed some promise as silylating solvent for micro samples, a time and temperature study was conducted to determine the optimum conditions for BSTFA and dichloroethane. The results are given in Table V. The data show that the optimum silylating conditions for dichloroethane were heating at 150° for 3 h; however, the guanine peak was still lower than desired. Then, a study of different amounts of acetonitrile and dichloroethane as silylating solvent was made to obtain a better guanine peak and still have good chromatography. It was found that a 1:1 ratio of acetonitrile and dichloroethane gave a fairly good guanine peak and chromatography.

Optimum silylating conditions. Since the two solvents used in the analysis of micro samples had different optimum silylating conditions when used alone, it was necessary to determine the best conditions for the solvent combination. The results of this study showed that the best response was obtained at 150° for 30 min. The reproducibility of silylation was considered good. The following $RMR_{B./I.S.}$ values and standard deviations were found: U — 0.78 ± 0.03; T — 0.83 ± 0.07; C— 0.63 ± 0.03; A — 0.70 ± 0.03; and G — 0.50 ± 0.04. A typical chromatogram for a micro sample is shown in Fig. 9, and represents 30 ng of each base injected.

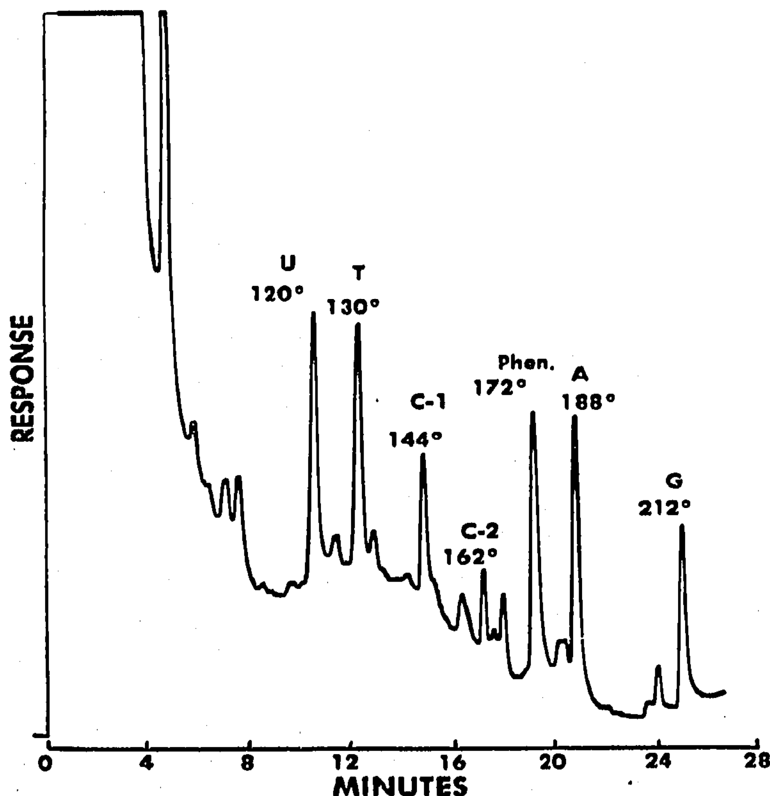

Fig. 9. GLC analysis of the purine and pyrimidine bases. Chromatographic conditions and column, see the legend to Fig. 7. Attenuation: 10 × 2. Silylation conditions: 500 ng each in reaction vial; 50 µl BSTFA; 25 µl acetonitrile; 25 µl dichloroethane; at 150° for 30 min; 6 µl (*ca.* 30 ng of each base) were injected.

CONCLUSIONS

BSTFA was found to be an excellent silylating reagent, giving good symmetrical peaks for all the bases and a good chromatographic baseline. The optimum silylating conditions for macro samples of the bases using BSTFA were found to be 0.2 ml BSTFA and 0.2 ml acetonitrile heated in a closed tube at 150° for 15 min. The reproducibility of silylation was excellent with the standard deviation ranging from 1.6 % for adenine to 2.7 % for cytosine. The silylated samples were stable for up to five days, however, most samples were chromatographed immediately after silylation to prevent possible deterioration.

For quantitative analysis of semimicro samples, the amount of silylating reagents had to be changed to 100 μl BSTFA and 100 μl acetonitrile; and the attenuation was lowered by a factor of four to achieve the best chromatography. Salts, used in drying certain reagents, did not have a great effect on the silylation or chromatography of the bases when the level of salt was that of each base. Water could also be present in small amounts without an adverse effect on the silylation and chromatography. But, because of the possible adverse effect salts or water could have on the silylation reaction and chromatography of the bases, care was taken to prevent the addition of salts or water to the reaction vial.

In the analysis of micro samples, it was necessary to change the silylating solvent from acetonitrile to a 1:1 ratio of acetonitrile and dichloroethane to obtain a good baseline and good peaks for all the bases. For micro samples, the best reaction conditions were the use of 50 μl BSTFA, 25 μl acetonitrile, and 25 μl dichloroethane heated in a closed "micro" tube at 150° for 30 min.

The GLC method for the purine and pyrimidine bases gave quantitative and reproducible results at each level; the silylated bases were stable with respect to time.

Investigations are in progress with this method and ion-exchange cleanup procedures for the analysis of hydrolyzed RNA and DNA samples. These data will be the subject of a separate paper.

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